

Fire S1. The expression patterns of zebrafish *eaf1* and *eaf2* during embryogenesis. (A) *eaf1* expression. (B) *eaf2* expression. Lateral view, dorsal to the right.

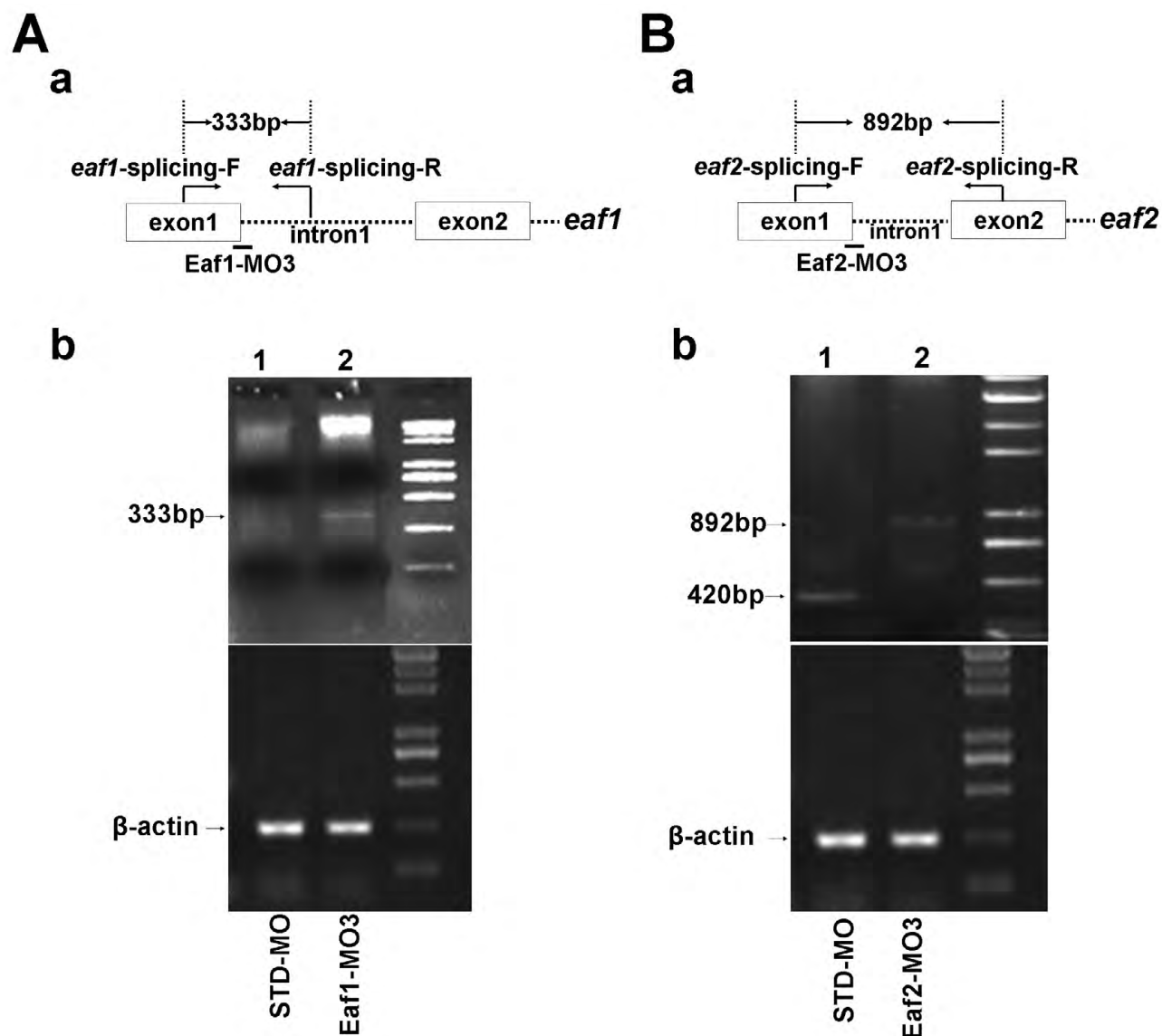


Fig. S2. Validation of Eaf1-MO3 and Eaf2-MO3. (A) (a) Schematic of *eaf1* exon-intron structure, Eaf1-MO3 targeting position and RT-PCR primer locations. (b) In STD-MO injection embryos, an expected 333 bp band could not be detected by RT-PCR, but in embryos with Eaf1-MO3 injection, a 333 bp band could be detected by RT-PCR. (B) (a) Schematic of *eaf2* exon-intron structure, Eaf2-MO3 targeting position and RT-PCR primer locations. (b) A 420 bp band was amplified from embryos injected with STD-MO by RT-PCR, but a 892 bp band was amplified from embryos injected with Eaf2-MO3, which contained intron 1 of *eaf2*. Embryos were collected at the bud stage for RNA extraction. Lane 1 is RNA from embryos injected with STD-MO, and lane 2 is RNA from embryos injected with Eaf1-MO3 (Ab) or Eaf1-MO3 (Bb).

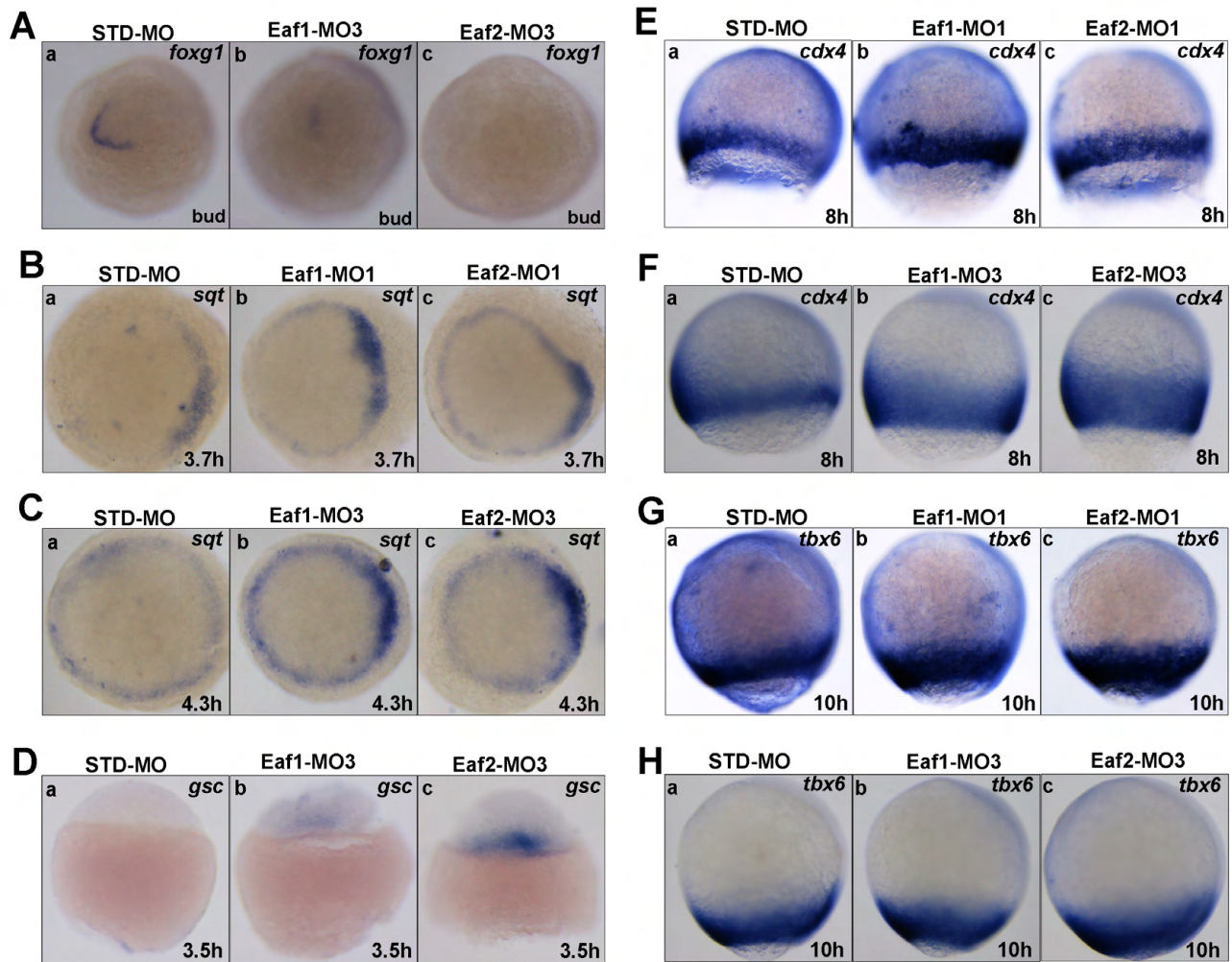


Fig. S3. Knockdown of zebrafish Eaf1 and Eaf2 causes defects in anterior neuroectoderm patterning and mesoderm patterning. (A) The expression of anterior neuroectoderm marker *foxg1*. (B-D) The expression of dorsal mesoderm marker genes *sqt* and *gsc*. (E-H) The expression of posterior ectoderm/mesoderm marker *cdx4* and ventral mesoderm marker *tbx6*. (A) Dorsal view, anterior to the left; (D) dorsal view; (B,C) animal view, dorsal to the right; (E-H) lateral view, dorsal to the right.

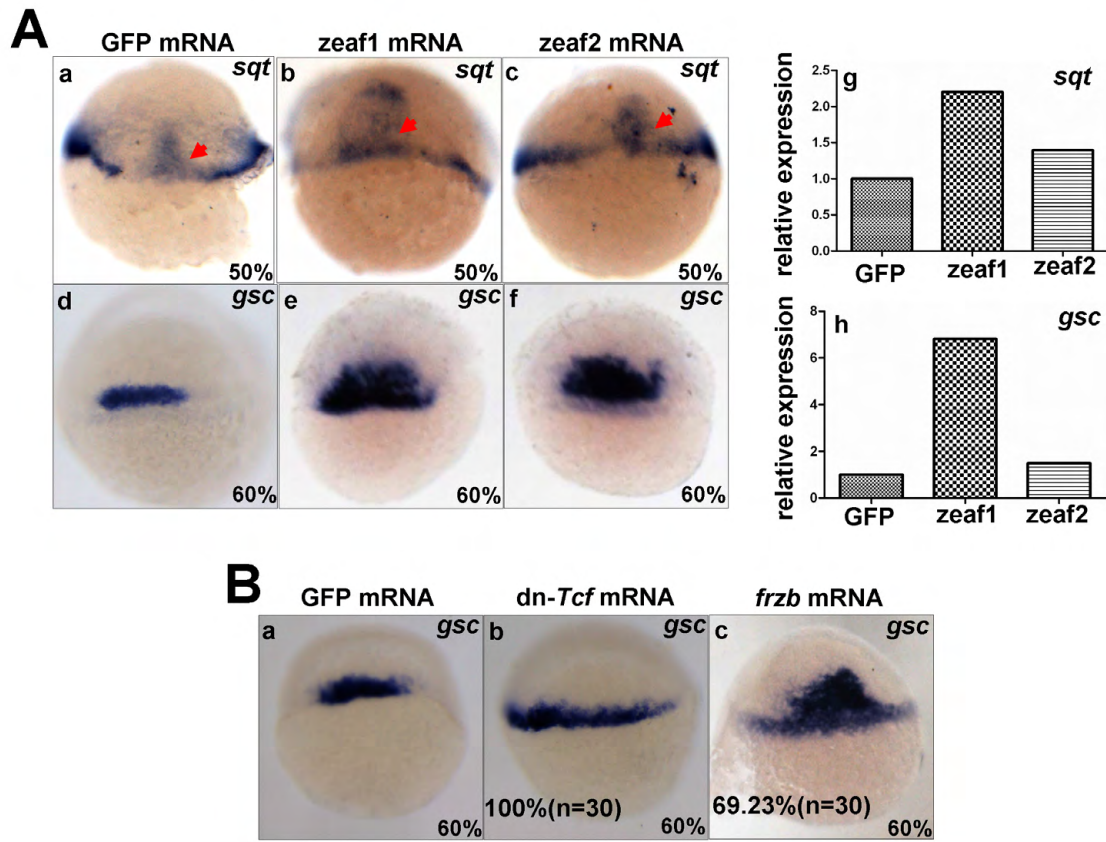


Fig. S4. Ectopic expression of zebrafish Eaf1 and Eaf2 increases the expression of *sqt* and *gsc*. (A) At the gastrula stage, dorsal marker genes *sqt* (a-c) and *gsc* (d-f) displayed expanded expression, particularly in the ventral domain. qRT-PCR was used to measure expression of *sqt* (g) and *gsc* (h) in these embryos. (B) In embryos injected with dn-*Tcf* mRNA (b) or *frzb* mRNA (c), the expression of *gsc* expanded ventral-laterally at the gastrula stage, compared with embryos injected with *GFP* mRNA (a). Dorsal views.

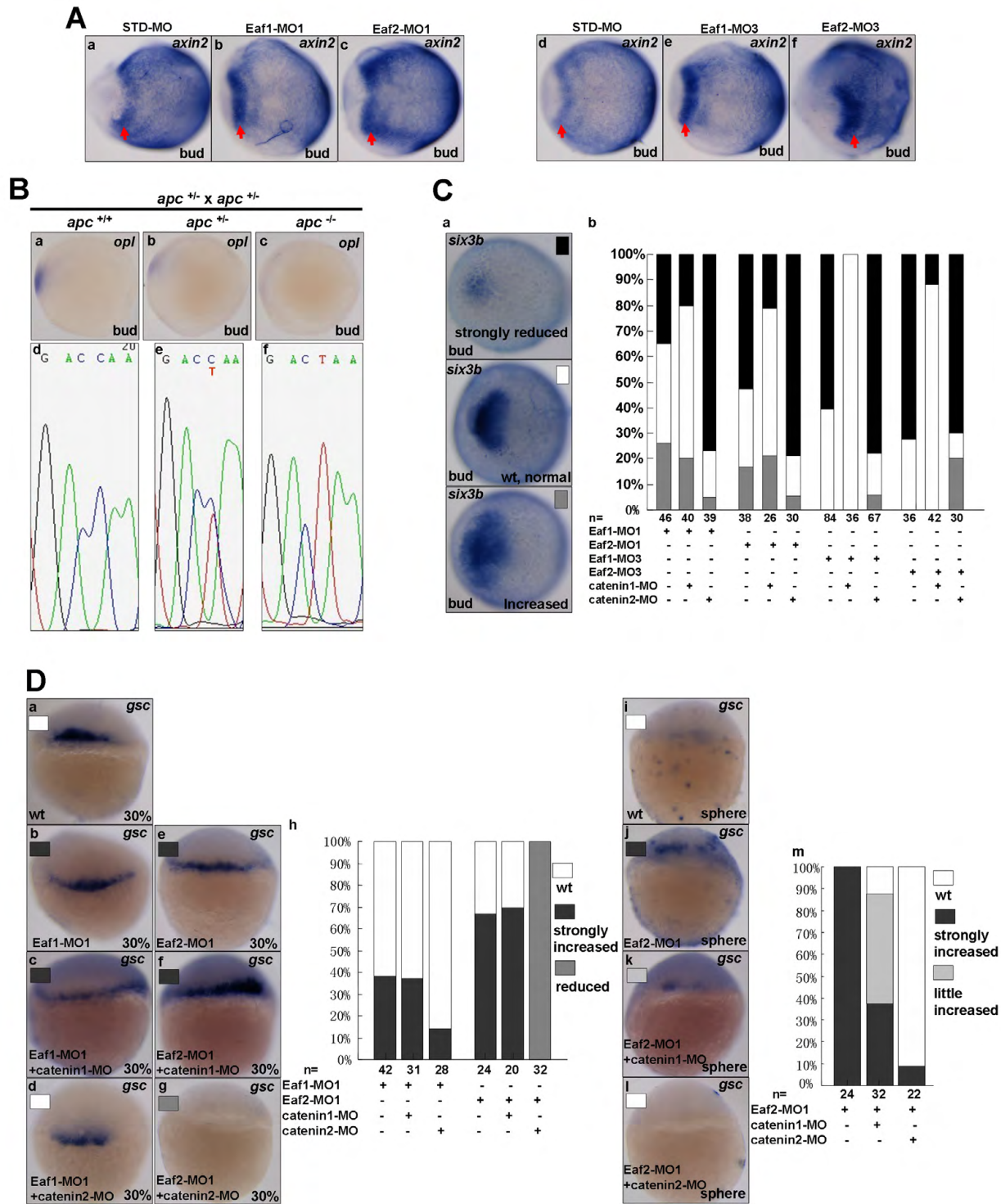


Fig. S5. *eaf1* and *eaf2* antagonize Wnt/ β -catenin signaling. (A) The expression of *axin2*, a bona fide direct target of Wnt/ β -catenin signaling at bud stage, was enhanced after knockdown of *eaf1* (b,e) or *eaf2* (c,f) (red arrows). (B) The offspring of *apc*^{+/-} × *apc*^{+/-} were genotyped by sequencing the mutant allele DNA fragment amplified from genomic DNA extracted from WISH-stained embryos. The embryos with normal *opl* expression contain the wild-type allele (C/C) (a,d), whereas the embryos with reduced *opl* expression contain the heterozygous allele (C/T) (b,e) and the embryos with strongly reduced *opl* expression contain the homozygous mutated allele (T/T) (c,f). (C) β -catenin1-MO, but not β -catenin2-MO, partially rescued the anterior brain defects in Eaf morphants. (D) At the 30% gastrula stage, β -catenin2-MO partially reduced the increased *gsc* expression associated with Eaf morphants (a-h); both β -catenin1-MO and β -catenin2-MO partially reduced the increased *gsc* expression of Eaf2-MO1 morphants at the sphere stage (i-m), and β -catenin2-MO was more effective (m). (A-C) Dorsal view, anterior to the left; (Da-g,i-l) dorsal view.

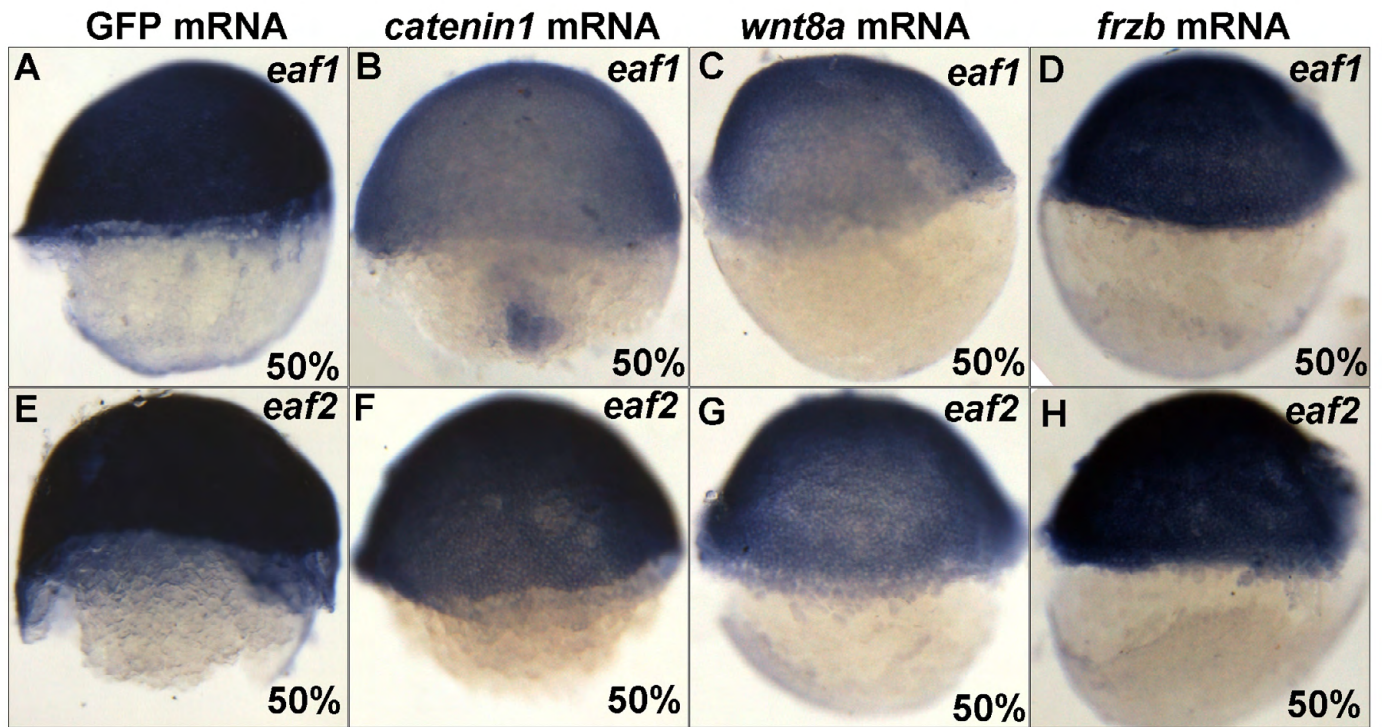


Fig. S6. The regulation of zebrafish *eaf1* and *eaf2* by Wnt/ β -catenin signaling. The expression of *eaf1* and *eaf2* is downregulated in embryos with ectopic expression of active β -catenin 1 mRNA (**B,F**) or *wnt8a* mRNA (**C,G**) as compared with embryos injected with *GFP* mRNA (**A,E**), but was maintained in embryos injected with *frzb* mRNA (**D,H**). Lateral views, dorsal to the right.

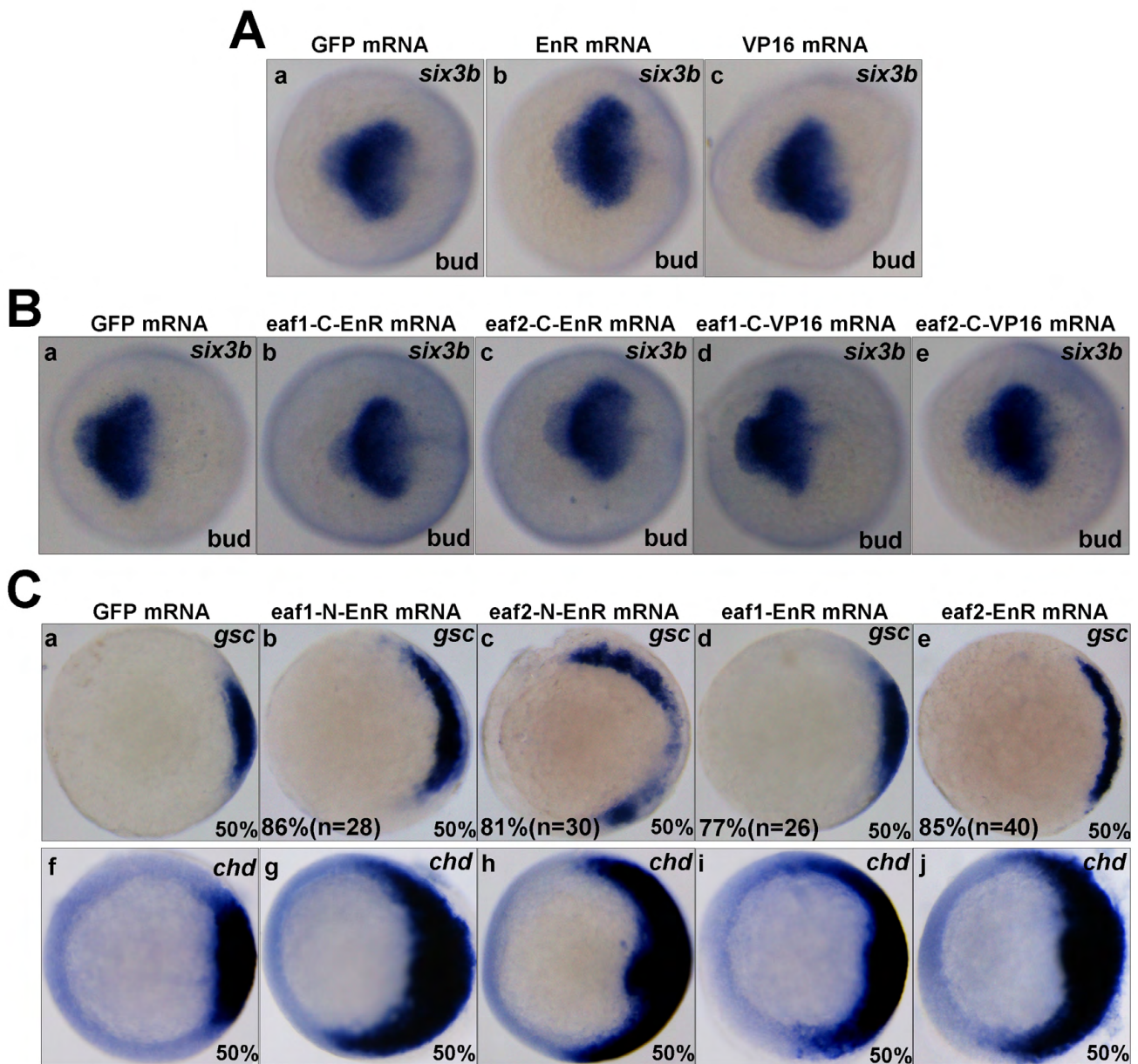


Fig. S7. VP16 and EnR are not functional *in vivo* and Eaf1/2 act as transcriptional repressors. (A,B) *Six3b* staining indicated normal patterning of anterior neural ectoderm in embryos injected with mRNA of the engrailed domain (EnR) or the VP16 domain (A), and in embryos injected with EnR or VP16 fused with the C-terminus of Eaf1 or Eaf2 (B). (C) In the embryos injected with *eaf1/2-EnR* or *eaf1/2-N-EnR* mRNA, the expression of the dorsal marker genes *gsc* and *chd* displayed similarly expanded patterns. (A,B) Dorsal views, anterior to the left; (C) animal views, dorsal to the right. All mRNA injections were 50-100 pg/embryo.

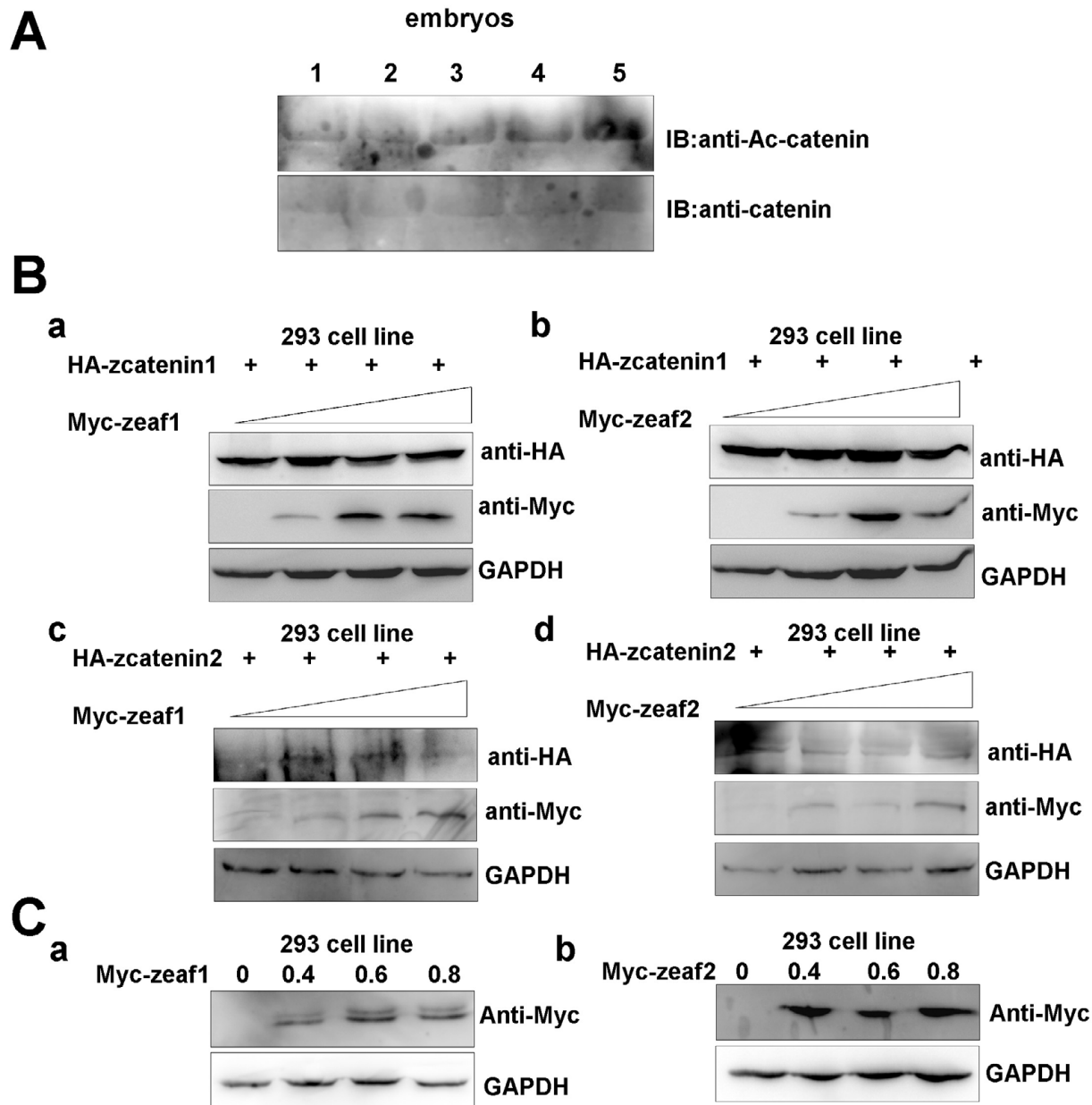


Fig. S8. Zebrafish Eaf1 or Eaf2 does not affect the protein level of either β -catenin 1 or β -catenin 2. (A) Western blot analysis for total β -catenin and active β -catenin protein (Ac-catenin) in embryos injected with STD-MO (lane 1), Eaf1-MO1 (lane 2), Eaf2-MO1 (lane 3), Eaf1-MO3 (lane 4) or Eaf2-MO3 (lane 5) (8 ng/per embryo). (B) The co-transfection of increased amounts of *eaf1* or *eaf2* together with β -catenin 1 and β -catenin 2 did not change the protein level of β -catenin 1 and β -catenin 2. (C) Western-blot analysis confirmed ectopic expression of zebrafish Eaf1 and Eaf2.

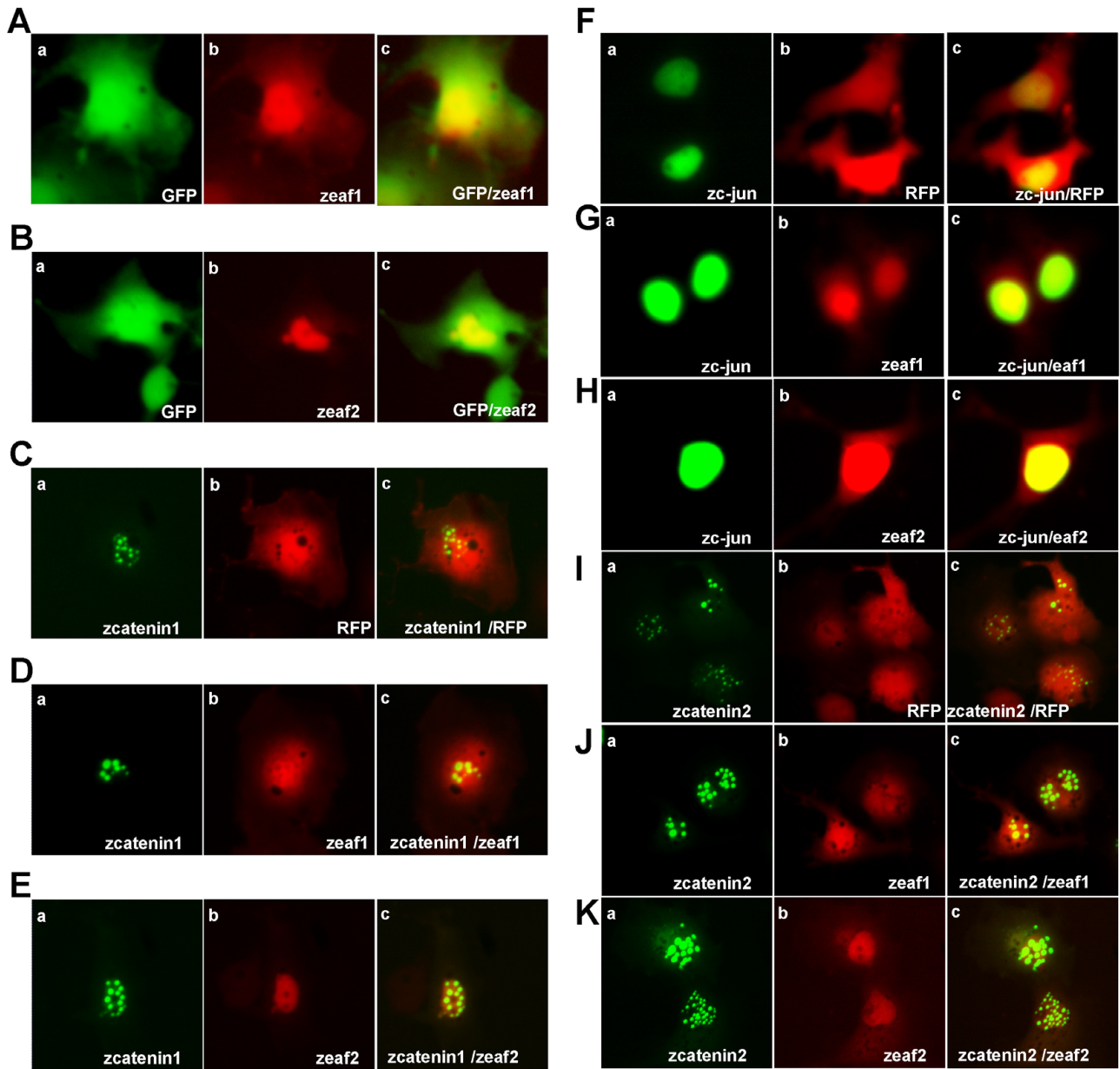


Fig. S9. Zebrafish Eaf1 or Eaf2 colocalizes with zebrafish β -catenin 1, β -catenin 2 and c-Jun. Cos-7 cells were transfected with: (A) empty GFP together with *eaf1*-RFP; (B) empty GFP together with *eaf2*-RFP; (C) empty RFP together with β -catenin1-GFP; (D) β -catenin1-GFP together with *eaf1*-RFP; (E) β -catenin1-GFP together with *eaf2*-RFP; (F) *c-jun*-GFP together with empty RFP; (G) *c-jun*-GFP together with *eaf1*-RFP; (H) *c-jun*-GFP together with *eaf2*-RFP; (I) β -catenin2-GFP together with empty RFP; (J) β -catenin2-GFP together with *eaf1*-RFP; (K) β -catenin2-GFP together with *eaf2*-RFP. The cells were photographed under a fluorescence microscope 24 hours after transfection.

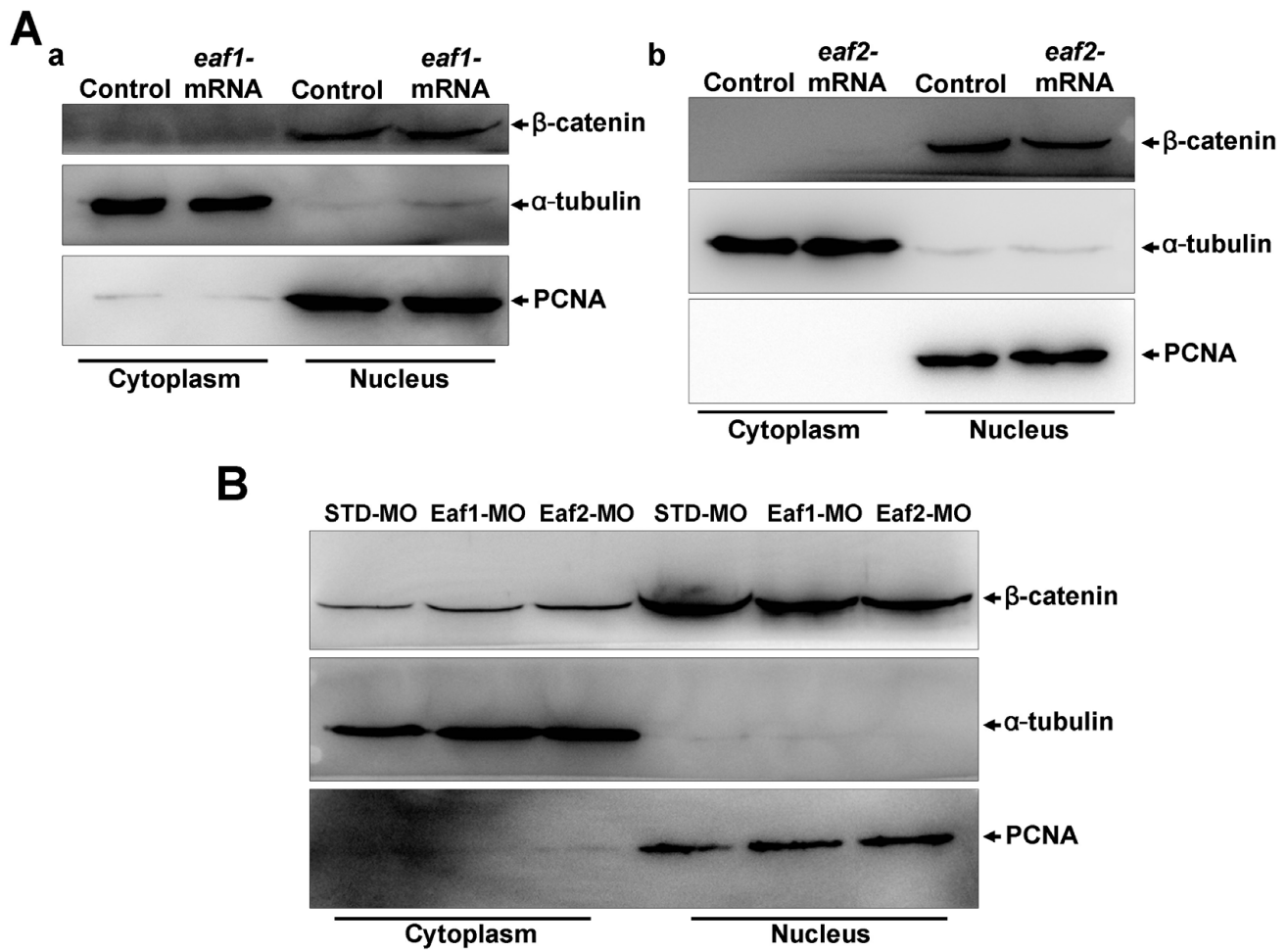


Fig. S10. Ectopic expression or knockdown of zebrafish *eaf1* or *eaf2* in embryos at the gastrula stage does not change cytoplasmic-nuclear translocation of β -catenin. (A) Ectopic expression of *eaf1* (a) or *eaf2* (b) did not change cytoplasmic-nuclear translocation of β -catenin. (B) Knockdown of *eaf1* or *eaf2* did not change cytoplasmic-nuclear translocation of β -catenin.

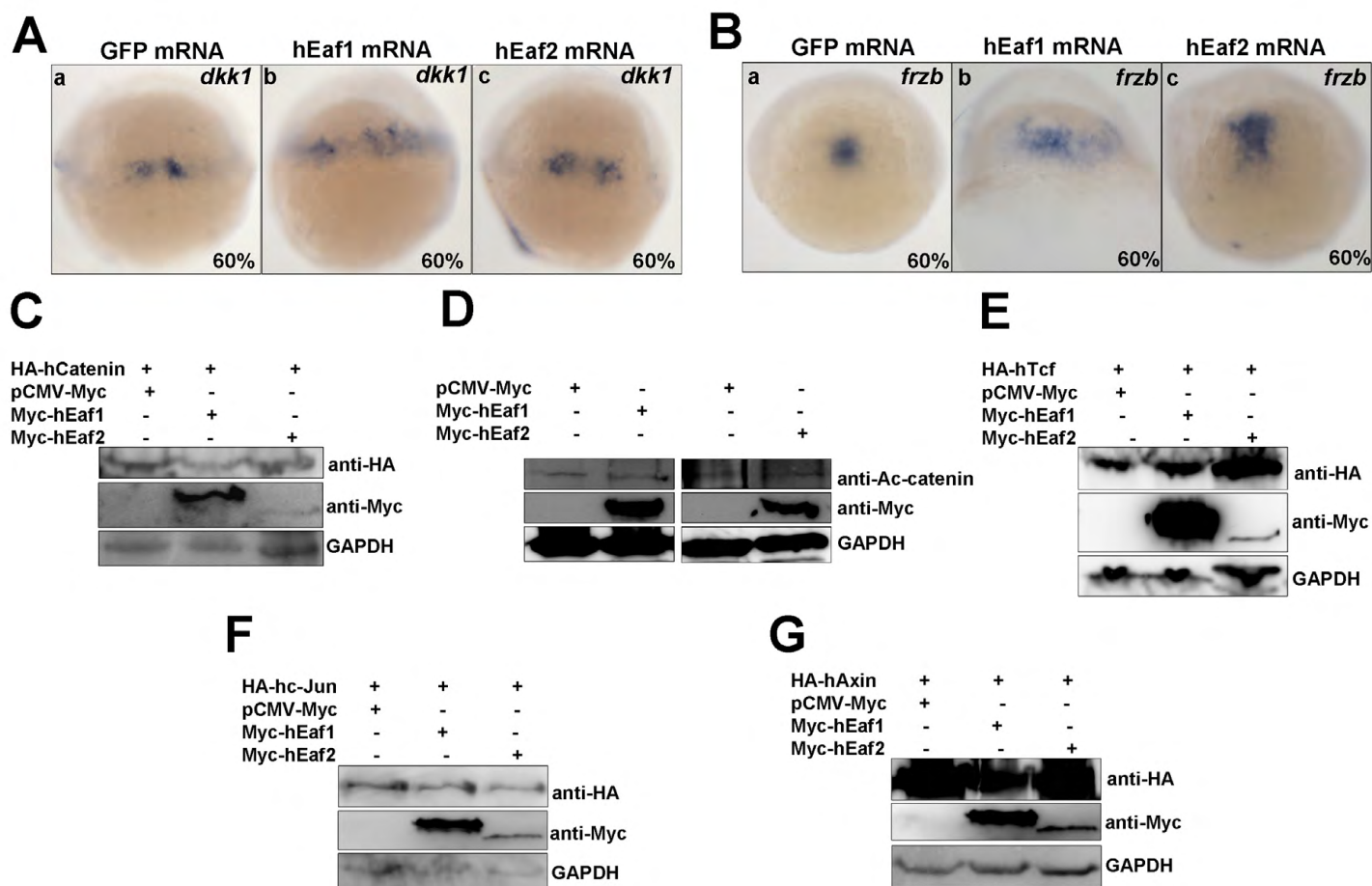


Fig. S11. Human Eaf function is evolutionary conserved in regulating Wnt/ β -catenin signaling. (A,B) In zebrafish embryos injected with human *EAF1* or *EAF2* mRNA, *dkk1* (A) and *frzb* (B) displayed enhanced expression. (C) Overexpression of human *EAF1* or *EAF2* did not affect co-transfected human β -catenin stability. (D) Overexpression of human *EAF1* or *EAF2* did not change the endogenous active β -catenin protein level. (E-G) Ectopic expression of human *TCF4* (E), human *c-JUN* (F) and human *AXIN* (G) after co-transfection with human *EAF1* or *EAF2* into the HEK 293T cell line.

Table S1. Primers used for qRT-PCR, probe amplification, splice MO validation, cDNA and promoter cloning

Primer	Sequence (5'-3')
sqtFq	GCTGGTGGTGGAAAGTGAAC
sqtRq	CCTTCACACCGATAAGCGTTG
gscFq	GCAAGAGACGACACCGAAC
gscRq	TGAACCAAACCTCTACCTTCTC
18sFq	GAGAAACGGCTACCACATCC
18sRq	CACCAGACTTGCCCTCCAA
c-mycFq (human)	GGCGAACACACAACGTCTTGGA
c-mycRq (human)	CTTACGCACAAGAGTTCCGTAGC
18sFq (human)	TCAACTTTCGATGGTAGTCGCCGT
18sRq (human)	TCCTTGGATGTGGTAGCCGTTTCT
cdx4-probe-F	CGTCCATGAGGAACATACAGC
cdx4-probe-R	CAAGAGCCTCCAGCATTTTCG
opl-probe-F	GGCGAAGTTACAGACAGA
opl-probe-R	GACATGACCGTATTGCTC
six3b-probe-F	TTTGGTCGTTGCCCGTAG
six3b-probe-R	CGTGATGCTGAAGCCTGT
eaf1-splicing-F	TGGTGAATTCAATGTGACGCGCAG
eaf1-splicing-R	GATTTGTTGGTGTGCGAATGTGGT
eaf2-splicing-F	AGAAGTGAGGCGTCTATTTCTGCC
eaf2-splicing-R	CTTGACCGCAATGTTGTTGCTGAG
zf-catenin2-F	ATATCTCTAGAAGGATTGACGCAACGATGGCTAG
zf-catenin2-myc-R	ATATCGCGGCCGCGTCCTTCGCTCAGCAGCTCTCTA-3
zf-catenin1-F	ATATCGAATTCGGTCTAGATGGCTACCCAGTCTGACTTGA

zf-catenin1-myc-R	ATATCGCGGCCGCTTACAGATCGGTGTCAAACCAGG
zf-c-jun-F	ATATCGAATTCGGTCTAGACCTTCTATGTCTACCAAGATGG
zf-c-jun-myc-R	ATATCGCGGCCGCGTCCTTCGGCTCTCCTCAGAAG
apc-sequencing-F	CTACCCAACTTTACCTATATCAG
apc-sequencing-R	GACTCTCAAAACTGTCAAGGG

Primers are zebrafish, except where marked for human.

Table S2. Morpholino sequences

Morpholino	Sequence (5'-3')
Eaf1-ATG-MO (Eaf1-MO1)	GCGGCGGGTTCGAGCTGCCGTTTCAT
Eaf2-ATG-MO (Eaf2-MO1)	ATGCTGTTCCATTTCATTCTAATCCA
Eaf1-splice-MO (Eaf1-MO3)	GTCTCTCTTGATGGACTCACATCTG
Eaf2-splice-MO (Eaf2-MO3)	AAAAGATGCAACTTACATCGTACCG
β -catenin1-MO	CTGGGTAGCCATGATTTTCTCACAG
β -catenin2-MO	CCTTTAGCCTGAGCGACTTCCAAAC